Exhibit C

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Synergistic Protection of Mice against Plague with Monoclonal Antibodies Specific for the F1 and V Antigens of *Yersinia pestis*

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Monoclonal antibodies specific for *Yersinia pestis* V antigen and F1 antigen, administered singly or in combination, protected mice in models of bubonic and pneumonic plague. Antibodies showed synergy when administered prophylactically and as a therapy 48 h postinfection. Monoclonal antibodies therefore have potential as a treatment for plague.

Yersinia pestis, the causative agent of plague, has accounted for the deaths of millions of people throughout recorded history. The second pandemic (the Black Death) is thought to have killed an estimated 17 to 28 million Europeans between the 14th and 17th centuries. The third pandemic, believed to have started in the Yunan Province of China in the 1850s, has led to the worldwide spread of plague, which is now endemic to several regions, including Africa, India, and the southwestern states of the United States (25). Despite the current low incidence of plague, the bacterium resides in natural animal reservoirs, and regular, although relatively small, outbreaks of plague occur (7, 19, 27). Improvements in transport links between areas of endemicity and large population centers bring with them the potential for large-scale plague outbreaks, highlighted by the recent outbreak in India (33). There is therefore a need for effective disease surveillance to reduce the risk of plague transmission to new areas and subsequent outbreaks of disease. Vaccination is recommended for research scientists and other professionals who come into contact with the bacterium, but fast-acting treatments are also required for individuals exposed to *Y. pestis* in areas of endemicity or through their work. In addition, after a major outbreak, there would be a need to protect health care workers and first responders.

At present, protection against plague can be mediated through vaccination or antibiotic treatment. Antibiotics are used both to treat plague victims and as prophylaxis to control the spread of the disease (25). The incidence of antibiotic resistance in *Y. pestis* is low, but recent plague isolates in Madagascar have been found to have multiple drug resistance, conferred by a transferable plasmid (10, 11). Although the bacteria were resistant to the frontline antibiotics streptomycin and tetracycline, they were susceptible to additional antibiotics.

Existing plague vaccines include killed whole-cell preparations, and efforts to develop new vaccines are in progress (39). Problems associated with whole-cell vaccines include relatively low levels of protection, adverse side effects, slow time to immunity, and a need for regular booster immunizations (30). Although whole-cell vaccines are thought to be effective against the most common form of plague (bubonic plague), which develops following a bite from an infected insect, their efficacy against pneumonic plague has been questioned. Consequently, whole-cell vaccines are no longer licensed for use in the United States. Next-generation plague subunit vaccines, based on the recombinant F1 and V (LcrV) antigen proteins derived from *Y. pestis*, are being developed. Immunization with either protein provides protection against pneumonic or bubonic disease in animal models of infection (12, 17, 39), but greater-than-additive protection is achieved when F1 and LcrV are combined, with protection against up to 10^9 median lethal doses (MLD) of *Y. pestis* reported (40). Such vaccines must be administered prior to exposure, and multiple doses are required. Although strategies to reduce the time to immunity and the number of vaccine doses have shown promise (41), it is unlikely that vaccination will provide postexposure protection against disease. There is therefore a need for alternative fast-acting antiplague treatments to provide rapid protection, particularly to combat drug-resistant strains of *Y. pestis*.

Because antisera have been used widely to treat a range of diseases caused by other pathogens (15), we considered monoclonal antibodies (MAbs) as a treatment for plague. Previously, F1-04-A-G1, a mouse MAb raised against F1, was shown to protect mice in models of bubonic and pneumonic plagues (1). Also, preliminary studies showed that an LcrV-specific MAb (MAb 7.3) protected mice in a bubonic plague model (13). In this study, we considered the prophylactic and therapeutic properties of MAb 7.3, when administered alone and in combination with F1-04-A-G1, to determine whether antibodies could be used as a postexposure therapy for plague.

MAb 7.3 and F1-04-A-G1 were purified by ammonium sulfate precipitation from hybridoma supernatants. An equal volume of saturated ammonium sulfate solution was added slowly to tissue culture supernatants, followed by overnight stirring at 4°C and then centrifugation at 3,000 × *g* for 30 min. The pellets were drained and resuspended in phosphate-buffered saline (PBS; GIBCO, Paisley, United Kingdom) in 0.1 volume of the original volume, which was then dialyzed against three changes of PBS. Disposable Econopak columns (BioRad, Hemel Hempstead, United Kingdom) were packed with protein G-Sepha-

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TABLE 1. Dose-dependent protection against bubonic plague with purified MAb 7.3

Concn of MAb 7.3 (μg)[a]	MLD[b]	No. of survivors/group	TTD (days)[c]
35	9.6	5/6	4.0
10.5	9.6	5/6	6.0
3.5	9.6	0/6	8.2 ± 1.1
0.7	9.6	1/6	4.8 ± 0.5
None	9.6	0/6	4.8 ± 0.3
35	96	3/6	6.3 ± 0.8
10.5	96	3/6	3.8 ± 2.7
3.5	96	1/6	6.4 ± 1.5
0.7	96	0/6	5.2 ± 0.4
None	96	0/6	4.1 ± 0.3

[a] Mab 7.3 administered i.p. 24 h before challenge.
[b] *Y. pestis* administered by s.c. injection in 100 μl of PBS.
[c] Values are means ± standard error.

rose beads (Sigma, Poole, United Kingdom), and antibody solution was passed through the column. The beads were washed with PBS, and then antibody was eluted with 50 mM glycine (pH 3) and stored in fractions containing 150 μl of Tris HCl (pH 9.1) per 3 ml of eluate. Protein fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10 to 15% Phastgels (Pharmacia, Milton Keynes, United Kingdom), and fractions containing antibody were dialyzed against three changes of PBS. Antibody concentration was determined by bicinchoninic acid assay (Perbio, Tattenhall, United Kingdom) with a bovine serum albumin standard as recommended by the manufacturers. Antibody purity was assessed by SDS-PAGE analysis.

Antibodies were tested in murine models of bubonic and pneumonic plagues. Six- to 8-week-old BALB/c mice were used (Charles River, Ltd., Margate, United Kingdom). Animal experiments were performed in accordance with United Kingdom legislation relating to animal experimentation (Animals [Scientific Procedures] Act 1986).

Mice received antibody by intraperitoneal (i.p.) injection in 100 μl of PBS prior to or after infection as indicated. *Y. pestis* strain GB, a fully virulent human isolate, with an estimated MLD of 1 CFU via the subcutaneous (s.c.) route (30), was used in all challenge experiments. In the bubonic plague model, mice received approximately 10 to 10^5 MLD resuspended in 100 μl of PBS, by s.c. injection. In the pneumonic plague model, mice were exposed to approximately 100 MLD of airborne bacteria, as described previously (42). Animals were checked at least twice daily, and deaths were recorded over a 14-day period.

MAb 7.3 protection. Mice were treated with purified MAb 7.3 24 h prior to challenge with 9.6 or 96 MLD of *Y. pestis*. As little as 3.5 μg of antibody protected mice and extended the mean time to death (TTD) of animals that died (Table 1). Greater survival was noted in groups given 10.5 or 35 μg than in those given 3.5 and 0.7 μg of MAb 7.3. The degree of protection was smaller in animals that received 96 MLD than in those injected with 9.6 MLD (50 and 83% were survivors, respectively). Therefore, protection against plague appeared to be proportional to the amount of antibody administered and was dependent on the challenge dose.

Five mice received 50 μg of MAb 7.3 in 100 μl of PBS by i.p. injection. Mice were tail bled regularly over a 7-day period, and levels of MAb 7.3 in serum were determined by anti-LcrV-specific enzyme-linked immunosorbent assay (ELISA), as described previously (13). These values were used to determine the time taken for the serum antibody levels to fall to half in individual animals; the average serum half-life was determined as 5.6 days. Because the serum antibody level 28 days after dosing was approximately 1.5 μg, five immunized animals were challenged with 18 MLD of *Y. pestis*. All MAb 7.3-treated animals survived, whereas six of six untreated mice died. This experiment demonstrated the potential for a single dose of antibody as a long-lasting prophylactic.

MAb 7.3 was administered −4, +24, +48, or +96 h relative to s.c. *Y. pestis* challenge. Protection was observed when antibody was given up to 48 h postinfection (Fig. 1A). Also, a statistically significant delay in the TTD was observed in the +96-h treatment group. One mouse in the +96-h treatment group had died prior to antibody administration, and the remainder displayed signs of plague indistinguishable from those in untreated control animals, suggesting that even when symptoms of plague are apparent, antibody therapy can delay death. Mice were treated with MAb 7.3 at −4, +24, +48, or +60 h relative to aerosol infection (Fig. 1B). Protection was seen in groups that received antibody 24 and 48 h after challenge. All mice treated at +60 h died, but a statistically significant delay in the TTD was observed, compared with that in untreated animals (Fig. 1B).

Combined F1-04-A-G1 and MAb 7.3 treatment. Because recombinant F1 and LcrV provide greater-than-additive protection when administered as a subunit vaccine in mice (40), we tested whether this was true for F1- and LcrV-specific antibodies. F1-O4-A-G1 was administered i.p. singly (100 μg) or in combination with MAb 7.3 (35 μg) in 100 μg of PBS 4 h prior to aerosol challenge with 88 MLD of *Y. pestis*. The various treatments protected mice against plague as follows. Out of each group of 10 mice, there were 0 survivors when treated with PBS alone, 9 survivors when treated with F1-04-A-G1, 10 survivors when treated with MAb 7.3, and 9 survivors when treated with F1-04-A-G1 plus MAb 7.3. Therefore, we confirmed the prophylactic properties of F1-04-A-G1 in the pneumonic plague model (1). MAb 7.3 was less effective as a treatment against s.c. *Y. pestis* challenge than aerosol challenge (Fig. 1), at least for the doses selected for each route; therefore, the bubonic plague model was chosen for further coadministration studies to test for antibody synergy. First, antibodies were tested as a pretreatment against challenge with approximately 50 to 10^5 MLD of *Y. pestis* GB (Table 2). Surprisingly, protection was observed at all challenge doses; breakthrough was expected at challenge doses greater than 100 MLD (Table 1) (1). Next, we tested the combined antibody treatment as a plague therapy. Mice that received the antibody cocktail 48 h after challenge with 91 MLD were protected better than animals that received single-antibody therapy (Fig. 2). The data suggest that MAb 7.3 and F1-04-A-G1 act synergistically as a pretreatment and as a therapeutic in our plague models.

Concluding remarks. We have demonstrated that MAbs specific for *Y. pestis* surface proteins can be used as a therapy for the treatment of plague. Mabs 7.3 and F1-04-A-G1 were more effective as a therapy when combined than as single

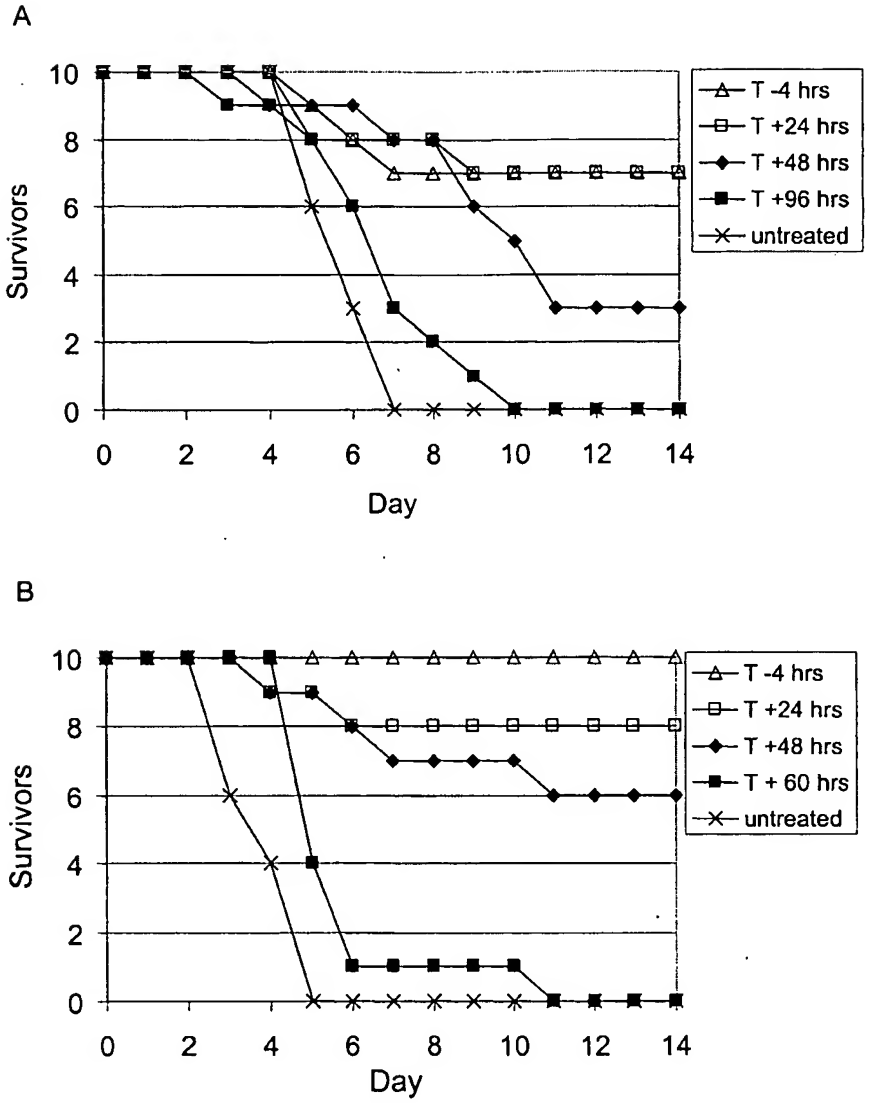

FIG. 1. Therapeutic MAb 7.3 treatment of mice challenged with *Y. pestis* via the s.c. (A) and aerosol (B) infection routes. Mice received 35 μg of MAb 7.3 in PBS by i.p. injection 4 h before or up to 96 h after challenge with 46 (A) or 88 (B) MLD of *Y. pestis*. Deaths were recorded over a 14-day period. A statistically significant delay in TTD was observed in animals treated with MAb 7.3 at 48 h ($P < 0.01$) and 96 h ($P < 0.05$) post-s.c. challenge and 60 h ($P < 0.05$) post-aerosol challenge by Student's *t* test analysis (Microsoft Excel software).

treatments, when administered up to 2 days after s.c. *Y. pestis* challenge. Together, the antibodies protected mice against an s.c. challenge of 9.1×10^4 MLD when administered as a pretreatment. The data presented here mirror observations that LcrV and F1 provide greater-than-additive protection when included in plague subunit vaccines (12, 40). Vaccine-mediated protection correlates with high specific polyclonal antibody titers to F1 and LcrV (43), which agrees with our observation that the degree of protection is proportional to the amount of protective antibody administered (Table 1).

Passive transfer of LcrV-specific polyclonal antiserum protected mice against plague, and the protective epitopes were assigned to region 168 to 275 (21). Similarly, we have mapped MAb 7.3 binding to a conformational epitope between amino

TABLE 2. Enhanced protection with F1-04-A-G1 and MAb 7.3 as a pretreatment against s.c. challenge

Antibody treatment[a]	*Y. pestis* challenge MLD[b]	No. of survivors/group
Untreated	46	0/6
F1-04-A-G1 + Mab 7.3	91	6/6
	9.1×10^2	6/6
	9.1×10^3	5/6
	9.1×10^4	6/6

[a] Mice were immunized i.p. with 35 μg of MAb 7.3 and 100 μg of F1-04-A-G1 in PBS.

[b] Plague challenge (s.c.) 4 h after antibody administration.

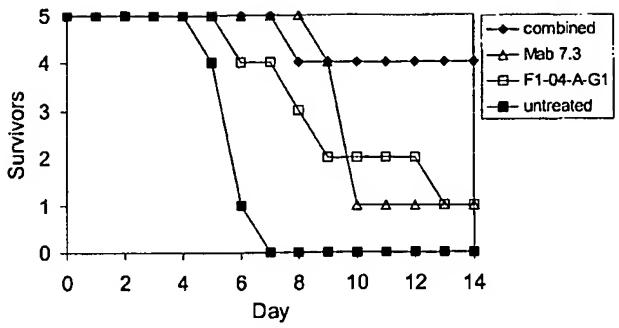

FIG. 2. MAb 7.3 and F1-04-AG-1 display synergy when administered postinfection. Mice were challenged s.c. with 91 MLD of *Y. pestis* and treated 48 h after plague challenge with MAb 7.3 (35 μg) or F1-04-A-G1 (100 μg) individually or together. Deaths were recorded over a 14-day period.

acids 135 to 275 of LcrV (13). Therefore, this central region of LcrV appears to be a good target for plague-protective antibodies. To date, epitope mapping studies have not been conducted with F1-04-A-G1.

LcrV has a key role in type III secretion (TTS) by *Yersinia* spp., a process that allows the injection of a set of effector proteins directly into the cytosol of eukaryotic target cells upon intimate contact (4, 14, 28, 29). The effector proteins (termed "Yops") have a range of functions that promote the killing of phagocytic host cells. Protective polyclonal antisera inhibited *Yersinia* TTS in HeLA cell cytotoxicity experiments, and LcrV was detected at the bacterial surface prior to contact with eukaryotic cells by confocal microscopy analysis (26). A similar study showed that MAb 7.3, but not other nonprotective Mabs, protected J774 macrophage-like cells against *Yersinia*-mediated killing (37). Antiserum raised against the LcrV homologue of *Pseudomonas aeruginosa* (PcrV) protected mice in a lung infection model, antiserum inhibited TTS-mediated cytotoxicity of J774 cells (9, 31), and anti-PcrV F(ab')2 fragment provided therapeutic protection in a model of disease (32). However, other studies did not show a correlation between protective LcrV-specific polyclonal antiserum in cytotoxicity assays (8). LcrV is also reported to have immunomodulatory properties (20, 22, 34, 38), so it remains a possibility that antibodies inhibit both TTS as well as the anti-inflammatory properties of *Y. pestis*, by blocking the interaction of secreted LcrV with an unidentified eukaryotic receptor.

F1 is expressed optimally at 37°C, is thought to inhibit phagocytosis through the formation of a capsule-like structure on the bacterial surface, and is an effective plague vaccine (2, 6, 12, 36). A recent report showed that an isogenic F1 plague mutant has impaired resistance to phagocytosis by J774 cells (6). Also, a virulence plasmid-cured strain, deficient for TTS, was less resistant to phagocytosis, and an additive effect was seen with the double mutant (F1-negative, plasmid-cured strain). It was proposed that the TTS system and F1 capsule synthesis contribute in different ways to maintain the extracellular lifestyle of *Y. pestis* (6). The fact that we have targeted both the TTS system and the F1 capsule might explain the high level of protection achieved with MAbs 7.3 and F1-04-A-G1.

A number of strategies can be used to generate clinically useful antibodies (3). For example, the specificity of animal antibodies can be transferred to a human antibody framework, a process termed "humanization" (35, 44), or animal antibodies can be chemically treated to improve their therapeutic properties (18). Alternatively, antibodies can be generated from naïve human single-chain antibody libraries (5, 16, 24) or from immunized transgenic animals that express a human antibody repertoire (23). Our findings have highlighted the benefits of combining antibodies specific for LcrV and F1. The next challenge will be to identify further targets for antibody intervention and to generate antibodies that are suitable for clinical use as a fast-acting pretreatment or postexposure therapy for plague.

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